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(54) **Title:** FUSION PROTEINS AND METHODS OF CLEAVAGE OF SUCH PROTEINS

(57) **Abstract:** The invention provides a method of processing recombinant proteins by using aspartate specific proteases.

FUSION PROTEINS AND METHODS OF CLEAVAGE OF SUCH PROTEINS.

FIELD OF THE INVENTION

The invention provides the construction of fusion proteins and cleavage of such fusion proteins in a specific manner by using caspases.

BACKGROUND OF THE INVENTION

Recombinant production of peptides and proteins sometimes requires fusion of peptide or proteins in order to achieve certain properties of the desired protein. The present invention provides a method of cleaving such fusion proteins and suitable cleavage sites.

Aspartate specific proteases are known in the art. Among these the caspases and the caspase activator Granzyme B are among the best described. These enzymes are all among the most specific proteolytic enzymes known to date due to their extended substrate recognition site. The caspases are cystein dependent aspartate specific proteases, which are well known for the ability to act as highly specific restriction proteases in vivo where they cleave a small subset of proteins in order to promote the development of the morphology characteristic of cells undergoing programmed cell death or apoptosis. Different Asp specific proteases are known in the art and described in (Thornberry Chem Biol. 1998; 5:R97-103.; Stennicke & Salvesen Biochim. Biophys. Acta 1998;1387:17-31). The different proteases have identical primary specificities, but very different secondary preferences and thus allows for the selection of that specific enzyme and matching cleavage site motif which ensures cleavage at the desired site while minimizing cleavage at secondary sites within the protein of interest while ensuring generation of the active *in vivo* form.

Numerous other fusion protein/processing systems are available today, however, most of these are highly sensitive to reducing conditions. The caspases, being thiol proteases, are on the other hand fully active under reducing conditions and remains active for some time in up to 4 M urea making them ideally suited for removal of tags or fusion partners in unfolded proteins.

SUMMARY OF THE INVENTION

The invention provides a method of producing a peptide comprising the steps of expression the peptide (e.g. as a fusion protein) which at the amino-terminal of the peptide has a sequence specific for an aspartate specific protease for the cleavage of fusion proteins and contacting the peptide with at least one aspartate-specific protease to liberate the peptide from the starting peptide – (e.g. the fusion protein).

In an embodiment of the invention the asparte specfic protease is selected from caspases 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, Granzyme B or CED-3

The invention provides the method as described above wherein the fusion protein is expressed in a host cell.

The invention provides the fusion protein between IL-21 and the sequence specific for asparte specific proteases.

The invention provides DNA encoding any of the fusion proteins described.

The invention provides a recombinant vector comprising the DNA encoding any of the proteins described.

The invention also provides a unicellular host organism containing a vector comprising the DNA encoding any of the proteins of above.

DEFINITIONS

In the context of the present invention IL-21 is defined as the sequence disclosed in WO00/53761 as SEQ ID No.:2. The invention also embraces functional derivatives and fragments thereof.

In the context of this invention "caspase" refers to any caspase regardless of origin or specificity and includes also proteases with caspase-like substrate specificity – such as Granzyme B.

The reference to positions Px and P'x refers to positions x aminoacids to the N-terminal and C-terminal respectively relative to the cleavage site (Schechter & Berger (1967) Biochem. Biophys. Res. Commun. 27, 157).

In the following the one-letter abbreviation for amino acid residues are used.

The terms peptide, polypeptide or protein are used interchangeably herein.

DESCRIPTION OF THE INVENTION

The protein polypeptides of the present invention, including full-length proteins, protein fragments (e.g. ligand-binding fragments), and fusion polypeptides can be produced in geneti-

cally engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed.(Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and Ausubel et al., ibid.

It is to be recognized that according to the present invention, when a cDNA is claimed as described above, it is understood that what is claimed are both the sense strand, the anti-sense strand, and the DNA as double-stranded having both the sense and anti-sense strand annealed together by their respective hydrogen bonds. Also claimed is the messenger RNA (mRNA) which encodes the polypeptides of the present invention, and which mRNA is encoded by the above-described cDNA. A messenger RNA (mRNA) will encode a polypeptide using the same codons as those defined above, with the exception that each thymine nucleotide (T) is replaced by a uracil nucleotide (U).

As mentioned, different asp specific proteases are known in the art and described. As a general rule caspases/granzyme B prefer the following motifs:

Protease	Preference
Caspase -2, - 3, - 7 and CED-3	DEXaaD-Zaa
Caspase -1, -4 and -5	(Y/F/W)VXaaD-Zaa
Caspase -6, -8, -9, -10 and Granzyme B	(I/L/V)EXaaD-Zaa

Where Xaa and Zaa can be any amino acid residue, with amino acids such as H, T and V being the preferred substituents for Xaa and S, T, G or A being preferred as Zaa, although large aromatic groups also are well tolerated (Stennicke et al. Biochem. J. (2000) *350*, 563-568).

In particular for Caspase I the most preferred P4 is Y or F

For Caspase 6 the most preferred P4 is Thr or Val;

For caspase 8 a preferred P4 is Leu:

For the position P1 all caspases prefer Asp.

The position P'1 in caspases are the preferred Gly, Ser, Ala, Phe or Tyr. The most preferred are Gly, Ser or Ala.

Preferred linkers include, but not limited to:
For Caspase 1: YVHD-A, LESD-N, WEHD-A
Caspase 3 and 7: DETD-S, DEVD-G, DMQD-N, DEVD-G and DGPD-G
Caspase 6: VEID-N, VEVD-A

The above linkers demonstrate selectivity in the linking moiety for cleavage by caspases.

In an embodiment of the invention the protease is a caspase 3.

In an embodiment of the invention the peptide is selected from the group consisting of aprotinin, tissue factor pathway inhibitor or other protease inhibitors, insulin or insulin precursors, human or bovine growth hormone, interleukine, glucagon, $Glucagon_{1-37}$ (oxyntomodulin), GLP-1, GLP-2, IGF-I, IGF-II, tissue plasminogen activator, transforming growth factor α or β, platelet-derived growth factor, GRF (growth hormone releasing factor), immunoglubolines, EPO, TPA, protein C blood coagulation factors such as FVII, FVIII, FV, FX, FIX or FXIII , exendin-3 or exentidin-4 or functional analogues of any of the above.

In another embodiment of the invention the peptide is an interleukine such as IL-20, IL-21, IL-28, IL-29 or IL-31 and the corresponding receptor molecules and soluble receptor molecules. In a specific embodiment of the invention the peptide is IL-21.

A specific embodiment is a fusion protein as above wherein the construct contains the linker sequence DEXaaD, or (Y/F/W)VXaaD, or (I/L/V)EXaaD, wherein Xaa is any aminoacid.
Another specific embodiment is a fusion protein as any of the above wherein the sequence added to the peptide is the sequence DEXaaD and wherein Xaa is any amino acid and the protease is caspase 2,3, 7 or CED-3.
Another specific embodiment is the fusion proteins as any of the above wherein the sequence added to the peptide is the sequence (Y/F/W)VXaaD and wherein Xaa is any amino acid and the protease is caspase 1, 4 or 5.

Another specific embodiment is a fusion protein as any of the above wherein the sequence added to the peptide is the sequence (I/L/V)EXaaD and wherein Xaa is any amino acid and the protease is caspase 6, 8, 9, 10 or Granzyme B.

Another specific embodiment is a fusion protein as any of the above wherein the sequence added is DETD, DEVD, DMQD, DEVD or DGPD and the protease is Caspase 3 or 7:

A specific embodiment of the invention is the recombinant IL-21 sequence in the C-terminal sequence and a construct recognisable by an aspartate specific protease as any of the above.

The above preferred embodiments are not to be construed as limiting to the invention. The selection of the linking moiety is a selection of specificity and kinetic considerations. The caspases has activity towards other motifs too, but with a lower selectivity and for some also a much lower specific activity.

Thus using a linker of this format any protein can be linked to the terminus of another protein to form a fusion protein. In a preferred embodiment this protein in IL-21.

The sequence preceding the protein or peptide of interest may be any sequence suitable for facilitating protein folding and/or purification. These sequences include, but are not limited to, myc-, T7-, HSV- , V5-, HA-, FLAG-, strep-tags, Glutathion transferase (GST), Green Fluorescent Protein (GFP), Thioredoxin (e.g. E.Coli TrxA), His_6 (-HHHHHH-) and variants thereof, chitin binding protein, maltose binding protein.

In a preferred embodiment of the present invention Caspase 3 is used due to its rare recognition site, however, other enzymes may be employed depending on the primary sequence of the protein of interest. This particular enzyme is characterised by a strict preference for Asp in P1 and P4 and Glu in P3 (previously described in Thornberry et al. J Biol Chem. 1997 Jul 18;272(29):17907-11.)

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989") DNA Cloning: A Practical Approach, Volumes I and II /D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait

ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984).

In an embodiment of the invention the peptide is aprotinin, tissue factor pathway inhibitor or other protease inhibitors, insulin or insulin precursors, human or bovine growth hormone, interleukin, glucagon, Glucagon$_{1-37}$ (oxyntomodulin), GLP-1, GLP-2, IGF-I, IGF-II, tissue plasminogen activator, transforming growth factor α or β, platelet-derived growth factor, GRF (growth hormone releasing factor), immunoglubolines, EPO, TPA, protein C, blood coagulation factors such as FVII, FVIII, FV, FX, FIX or XIII as well as exendin-3 or exentidin-4, and functional analogues of any of the above. In an embodiment of the invention the peptide is an interleukine such as IL-20, IL-21, IL-28, IL-29 or IL-31 and the corresponding receptor molecules and soluble receptor molecules. In a specific embodiment of the invention the peptide is IL-21.

In the present context, the term "functional analogue" is meant to indicate a protein with a similar function as the native protein. The protein may be structurally similar to the native protein and may be derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end of the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native protein or at one or several sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. Furthermore the protein may be acylated in one or more positions, vide WO 98/08871 which discloses acylation of GLP-1 and analogues thereof and in WO 98/08872 which discloses acylation of GLP-2 and analogues thereof. An example of an acylated GLP-1 derivative is Lys^{26}(N$^{\varepsilon}$-tetradecanoyl)-GLP-1$_{(7-37)}$ which is GLP-1$_{(7-37)}$ wherein the ε-amino group of the Lys residue in position 26 has been tetradecanoylated.

An insulin analogue is an insulin molecule having one or more mutations, substitutions, deletions and or additions of the A and/or B amino acid chains relative to the human insulin molecule. The insulin analogues are preferably such wherein one or more of the naturally occurring amino acid residues, preferably one, two, or three of them, have been substituted by another codable amino acid residue. Thus position 28 of the B chain may be modified from the natural Pro residue to one of Asp, Lys, or Ile. In another embodiment Lys at position B29 is modified to Pro; Also, Asn at position A21 may be modified to Ala, Gln, Glu, Gly, His, Ile, Leu, Met, Ser, Thr, Trp, Tyr or Val, in particular to Gly, Ala, Ser, or Thr and preferably to Gly. Furthermore, Asn at position B3 may be modified to Lys. Further examples of insulin analogues are des(B30) human insulin, insulin analogues wherein PheB1 has been deleted; insulin

analogues wherein the A-chain and/or the B-chain have an N-terminal extension and insulin analogues wherein the A-chain and/or the B-chain have a C-terminal extension. Thus one or two Arg may be added to position B1, or one Arg to position B30 and one Arg to position B31. Also, precursors or intermediates for other proteins may be treated by the method of the invention. An example of such a precursor is an insulin precursor which comprises the amino acid sequence B(1-29)-AlaAlaLys-A(1-21) wherein A(1-21) is the A chain of human insulin and B(1-29) is the B chain of human insulin in which Thr(B30) is missing. Combinations of the above analogues are also included. Thus the insulin analogue A21Gly- B30Arg–B31Arg is within the insulin analogues of the invention. Finally, the insulin molecule may be acylated in one or more positions, such as in the B29 position of human insulin or desB30 human insulin. Examples of acylated insulins are $N^{\epsilon B29}$-tetradecanoyl Gln^{B3} des(B30) human insulin), $N^{\epsilon B29}$-tridecanoyl human insulin, $N^{\epsilon B29}$-tetradecanoyl human insulin, $N^{\epsilon B29}$-decanoyl human insulin, and $N^{\epsilon B29}$-dodecanoyl human insulin.

"IL-21" is described in International Patent Application publication no. WO 00/53761, published September 14, 2000, which is hereby incorporated in this application in its entirety, discloses IL-21 (as "Zalpha11 ligand") as SEQ ID No. 2, which is hereby incorporated in this application in its entirety, as well as methods for producing it and antibodies thereto and a polynucleotide sequence encoding IL-21 as SEQ ID No. 1 in the aforementioned application. The invention comprises their orthologs comprising at least 70%, at least 80%, at least 90%, at least 95%, or greater than 95%. The present invention also includes the use of polypeptides that comprise an amino acid sequence having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the sequence of amino acid residues. Methods for determining percent identity are described below. The polypeptides of the present invention have retained all or some of the biological activity of the native counterparts. Some of the polypeptides may also have a biological activity which is higher than the biological activity of the native proteins.

The present invention embraces counterpart proteins and polynucleotides from other species ("species orthologs"). Of particular interest are polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primates. Species orthologs of the human protein can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. As used and claimed, the language "an isolated polynucleotide which encodes a polypeptide, said polynucleotide being defined by the sequences includes all allelic variants and species orthologs of this polypeptide.

The present invention also provides isolated protein polypeptides that are substantially identical to the protein polypeptides and their species orthologs. By "isolated" is meant a protein or polypeptide that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. The term "substantially identical" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence of the native proteins or species orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to the native protein, or its species orthologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-616 (1986) and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-10919 (1992). Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Variant polypeptides or substantially identical proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 1) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly- histidine tract, protein A, Nilsson et al., EMBO J. 4:1075 (1985); Nilsson et al., Methods Enzymol. 198:3 (1991), glutathione S transferase, Smith and Johnson, Gene 67:31 (1988), or other antigenic epitope or binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107 (1991). DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

Table 1

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid

	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methanoproline, cis-4-hydroxyproline, trans-4-hydroxyproline, Nmethylglycine, addo-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating nonnaturally occurring amino acid residues into proteins. For example, an in vitro system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Other methods are described by Schultz et al. J. Org. Chem., 2003, 68, 174-176 or Science 2003, vol 299, 640. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine scanning mutagenesis [Cunningham and Wells, Science 244: 1081-1085 (1989)]; Bass et al., Proc. Natl. Acad. Sci. USA 88:4498-4502 (1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., ligand binding and signal transduction) to identify amino

acid residues that are critical to the activity of the molecule. Sites of ligand-protein interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., Science 255:306-312 (1992); Smith et al., J. Mol. Biol. 224:899-904 (1992); Wlodaver et al., FEES Lett. 309:59-64 (1992). The identities of essential amino acids can also be inferred from analysis of homologies with related proteins.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer, Science 241:53-57 (1988) or Bowie and Sauer Proc. Natl. Acad. Sci. USA 86:2152-2156 (1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem.30:10832-10837 (1991); Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis, Derbyshire et al., Gene 46:145 (1986); Ner et al., DNA 7:127 (1988).

The present invention further provides a variety of other polypeptide fusions and related multimeric proteins comprising one or more polypeptide fusions. Polypeptide fusions can be expressed in genetically engineered cells. Auxiliary domains can be fused to the polypeptides to target them to specific cells, tissues, or macromolecules (e.g., collagen). For example, a IL-21 polypeptide or protein could be targeted to a predetermined cell type by fusing a polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A IL-21 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., Connective Tissue Research 34:1-9 (1996).

Using the methods discussed above, one of ordinary skill in the art can prepare a variety of polypeptides or allelic variants thereof and retain the properties of the wildtype protein. As expressed and claimed herein the language, "a polypeptide" includes all allelic variants and species orthologs of the polypeptide.

The protein polypeptides of the present invention, including full-length proteins, protein fragments (e.g. ligand-binding fragments), and fusion polypeptides can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multi-cellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed.(Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and Ausubel et al., ibid.

EXAMPLES

General procedure:

Step A:

1. Select the appropriate Caspase by determining the existence of secondary cleavage sites. This procedure may for a large part be done *in silico* as simple sequence analysis may exclude some candidate enzymes, however, most often only the absence of sites can be used for deciding on a particular enzyme while the presence of a site not necessarily warrants exclusion as the sequence may be unaccessible in the three-dimensional structure. In the case a potential cleavage site is present in the structure the accessibility may be probed by caspase treatment using elevated enzyme concentrations, i.e., a 1:20 or 1:50 ratio of caspase to product under conditions providing for optimal caspase activity (Stennicke & Salvesen. J.Biol.Chem. (1997) **272**: 25719-25723), in order to establish the level of aberrant cleavage.

2. Generate the appropriate fusion protein by combination of the optimal linker and fusion partner/tag. TAG – Caspase site – IL21. Once decision has been made on a particular enzyme the IL21 fusion protein may be generated by genetic engineering. A very simple example of such a fusion protein may be MHHHHHH-DEVD-IL21, which would allow for purification of the product by metal chelating chromatography and processing by caspase 3 or 7 according to the aforementioned caspase specificities.

3. Express protein in a suitable expression host system. Protocols for expression of Met-IL21 into inclusion bodies (IBs) in E.coli has previously been described (WO 00/53761 or WO 04055168) and this protocol may be utilized for the fusion protein as well. Following expression the cells are harvested by centrifugation and broken open by a suitable physical or chemical technique, e.g. sonocation or French press. The IBs and cell fragments are collected by centrifugation and the pellet washed repeatedly with a suitable buffer contain detergent for solubilization of cell fragments. The semi-pure IBs are then washed with water to remove any detergent residues before they are solubilized in a suitable denaturant, typically urea or guanidine. At this point the protein may be further purified using the affinity tag according to conventional procedures.

4. Activation of desired caspase by addition of reductive equivalents. An important step in the use of thiol proteases such as the caspases is the pre-activation of the enzymes by reduction. This is most easily done by addition of DTT to the enzyme stock solution to a final concentration of 10 mM, which will result in rapid activation of the enzyme at 37°C.

5. Cleavage of folded or unfolded. Once the fusion protein has been isolated it may be cleaved directly by the caspase in buffer containing 3-4 M Urea and EDTA or reducing equivalents. Alternatively, it may be refolded using the established procedure (ref.) and the pure refolded material may then be processed in a suitable buffer. The time required to complete the processing depends on the caspase concentration, buffer composition and the N-terminal sequence of the desired IL-21 protein.

6. Separation of TAG and unprocessed material from the product. To remove released tags and unprocessed material the material is simply passed through the affinity matrix and the flow-trough containing the released IL-21 is collected. To simplify the removal of the caspases there exists several options: a) they may be produced with the same tag as IL21 which allows for removal along with unprocessed material, b) they may be covalently immobilized on beads or c) a mixed bed column containing several affinity matrixes may be used to remove unprocessed material, tags and the enzyme in one step if different tags are used.

PHARMACOLOGICAL METHODS

Cell pellet from fermentation of E.Coli expressing CISNN116 (NT1-C3-hIL21) was resuspended in lysis buffer containing 2 tablets/L Complete™, 200 mg/L DNAse and 20 mg/L $MgCl_2$. Cells were subsequently opened using a cell dispruter running at 2000 Bar. Inclusion bodies were collected by centrifugation.

The inclusion bodies (10 g) were resuspended in 100 ml 6 M Guanidinium HCl, 100 mM Tris, 40 mM Dithiothreitol (DTT) at pH 8.0. The protein was refolded using a volumetric method, by slow addition of the resuspended and reduced inclusion bodies to 2 L of 0.9 M Arginine, 10 mM NaCl, 2 mM $MgCl_2$, 0.5 mM KCl, 2 mM $CaCl_2$, 1.5 mM DTT, 4 mM L-Cystine, 50 mM Tris, pH 7.5 at 15 °C.

After refoldning overnight, the protein was diluted 4 times in 25 mM Tris pH 7.5 and run on a TosoHaas sp 550c column, using a NaCl gradient as elution. Fractions containing the protein was pooled and run on a affinity column.

The buffer used for Caspase 3 proteolysis of the IL21 fusion protein was; 20 mM Tris, 0.3 M NaCl, 25 mM L-Histidine, 10 mM EDTA-Na^{2+}, 1 mM GSH, pH 7.0. The pooled fractions were preheated at 37 °C, prior to addition of Caspase 3. Proteolysis was run for 1 hour at 37 °C. Final concentration of tag-DEVD-hIL21 and Caspase 3 were 77 µM and 90 nM, respectively.

Maldi analysis showed 87 % completion of the reaction. The native hIL-21 was separated from tag-DEVD-hIL-21 fusion protein and other impurities using a Mono S column run in 25 mM Tris buffer pH 7.5 with a NaCl gradient as elution.

4 w/v % of Mannitol was added to the final fraction containing hIL-21 for stabilisation of the protein. Subsequent receptor assay (BaF assay) and DSC (differential scanning calorimetry) analysis showed that the hIL-21 produced from CISNN116 is equipotent with, and showed same melting temperature of 57 °C as previous purified batches of Met-hIL-21.

CLAIMS

1. A method for recombinant production of a peptide comprising the steps of expression the peptide as a fusion protein comprising a sequence specific for an aspartate specific protease and applying the protease to cleave the fusion protein.

2. The method of claim 1 wherein the asparte specific protease is selected from caspases 1, 2, 3, 4, 5 , 6, 7, 8, 9 or 10 or Granzyme B or CED-3

3. The method of claims 1-2 wherein the protease is a caspase 3.

4. The method of claims 1-3 wherein the peptide is selected from the group consisting of aprotinin, tissue factor pathway inhibitor or other protease inhibitors, insulin or insulin precursors, human or bovine growth hormone, interleukin, glucagon, $Glucagon_{1-37}$ (oxyntomodulin),GLP-1, GLP-2, IGF-I, IGF-II, tissue plasminogen activator, transforming growth factor α or β, platelet-derived growth factor, GRF (growth hormone releasing factor), immunoglubolines, EPO, TPA, protein C, blood coagulation factors such as FVII, FVIII, FV, FX, FIX or XIII , exendin-3, exentidin-4.

5. The method of claims 1-3 wherein the interleukine is selected from IL-20, IL-21, IL-28, IL-29 or IL-31 and the corresponding receptor molecules and soluble receptor molecules.

6. The method of claim 5, wherein the peptide is IL-21.

7. A fusion protein comprising a peptide as in claims 4-6, wherein the sequence comprised by the fusion proteins is recognisable by an aspartate specific protease.

8. A fusion protein of claim 7, wherein the peptide is IL-21.

9. The fusion protein of claims 7-8, wherein the fusion protein comprises the sequence DEXaaD, or (Y/F/W)VXaaD, or (I/L/V)EXaaD , wherein Xaa is any amino acid.

10. The fusion protein of claims 7-9, wherein the sequence comprised by the fusion protein is the sequence DEXaaD wherein Xaa is any amino acid, and the protease is caspase 2, 3, 7 or CED-3.

11. The fusion proteins according to any of the claims 7-9, wherein the sequence comprised by the fusion protein is the sequence (Y/F/W)VXaaD and wherein Xaa is any amino acid and the protease is caspase 1, 4 or 5.

12. The fusion protein according to any of the claims 7-9 wherein the sequence comprised by the fusion protein is the sequence (I/L/V)EXaaD and wherein Xaa is any amino acid and the protease is caspase 6, 8, 9, 10 or Granzyme B.

13. The fusion protein according to any of the claims 7-10 wherein the sequence added is DETD, DEVD, DMQD, DEVD or DGPD and the protease is Caspase 3 or 7:

14. A nucleic acid molecule encoding any of the fusion proteins described in claims 1-13.

15. A recombinant vector comprising the nucleic acid molecule encoding any of the proteins of claims 1-13

16. A unicellular host organism containing a vector comprising the nucleic acid molecules encoding any of the proteins of claim 1-13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000815

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P21/06 C07K14/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MERTENS N ET AL: "NEW STRATEGIES IN POLYPEPTIDE AND ANTIBODY SYNTHESIS: AN OVERVIEW" CANCER BIOTHERAPY AND RADIOPHARMACEUTICALS, LIEBERT, US, vol. 19, no. 1, February 2004 (2004-02), pages 99-109, XP008032571 figure 1; table 1 -----	1-16
P,X	WO 2004/074488 A (BIOTECNOL S.A; VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE;) 2 September 2004 (2004-09-02) figures 3a,3b; examples 1-5 -----	1-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 February 2005	25/02/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schmitz, T

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000815

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 00/61768 A (YEDA RESEARCH AND DEVELOPMENT CO. LTD; RUBINSTEIN, MENACHEM; LIU, BIAN) 19 October 2000 (2000-10-19) examples 8-11 page 3, line 23 - line 31 figure 8	1-16
X	WO 01/98455 A (SMITHKLINE BEECHAM CORPORATION; JOHANSON, KYUNG, O; KIRKPATRICK, ROBER) 27 December 2001 (2001-12-27) figure 10; examples 5-16	1-16
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X	WO 02/06458 A (CHEMICON INTERNATIONAL, INC) 24 January 2002 (2002-01-24) page 29, line 8 - page 30, line 9	1-3,15, 16
X	WO 00/73437 A (MERCK FROSST CANADA & CO; XANTHOUDAKIS, STEVEN; TAWA, PAUL; CASSADY, R) 7 December 2000 (2000-12-07) examples 1-9	1-3,15, 16
A	STENNICKE ET AL: "Biochemical characteristics of caspases-3, -6, -7 and -8" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 272, no. 41, 1997, pages 25719-25723, XP002146144 ISSN: 0021-9258 cited in the application the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2004/000815

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-6 may be interpreted as being to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK2004/000815

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